

Experimental modification of rat pituitary growth hormone cell function during and after spaceflight

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Hymer, W. C., R. E. Grindeland, T. Salada, P. Nye, E. J. Grossman, and P. K. Lane. Experimental modification of rat pituitary growth hormone cell function during and after spaceflight. *J. Appl. Physiol.* 80(3): 955-970, 1996.—Spaceflown rats show a number of flight-induced changes in the structure and function of pituitary growth hormone (GH) cells after in vitro postflight testing (W. C. Hymer, R. E. Grindeland, I. Krasnov, I. Victorov, K. Motter, P. Mukherjee, K. Shellenberger, and M. Vasques. *J. Appl. Physiol.* 73, *Suppl.*: 151S-157S, 1992). To evaluate the possible effects of microgravity on growth hormone (GH) cells themselves, freshly dispersed rat anterior pituitary gland cells were seeded into vials containing serum \pm 1 μ M hydrocortisone (HC) before flight. Five different cell preparations were used: the entire mixed-cell population of various hormone-producing cell types, cells of density $<$ 1.071 g/cm³ (*band 1*), cells of density $>$ 1.071 g/cm³ (*band 2*), and cells prepared from either the dorsal or ventral part of the gland. Relative to ground control samples, bioactive GH released from dense cells during flight was reduced in HC-free medium but was increased in HC-containing medium. *Band 1* and mixed cells usually showed opposite HC-dependent responses. Release of bioactive GH from ventral flight cells was lower; postflight responses to GH-releasing hormone challenge were reduced, and the cytoplasmic area occupied by GH in the dense cells was greater. Collectively, the data show that the chemistry and cellular makeup of the culture system modifies the response of GH cells to microgravity. As such, these cells offer a system to identify gravisensing mechanisms in secretory cells in future microgravity research.

microgravity; cell culture; cell gravisensing; growth hormone assays

IF AND HOW CELLS SENSE the low gravity of spaceflight has been thoughtfully considered, modeled, and debated for years. Many different types of cells have been exposed to microgravity (14); some show significant and repeatable changes (4-6, 19, 24). Although this area of investigation is only beginning, many believe that these kinds of studies will 1) eventually help to define the mechanisms that cells and organisms use to respond to this unique environment and 2) help to explain the well-documented changes in the musculoskeletal, immune, vascular, and endocrine systems of spaceflown animals and astronauts (11, 12). We studied growth hormone (GH) cell structure and function in three previous spaceflight experiments because pituitary GH participates in the regulation of these organ systems. We found that significant changes had occurred in the GH and prolactin (PRL) cells prepared from animals in microgravity for 7-14 days; interest-

ingly, many of these changes persisted for 2 wk postflight (15, 16).

This report describes changes in rat GH cells themselves during and after an 8-day spaceflight with a passive cell culture system. The most important changes found were those relating to 1) the amount and biological activity of GH released from cells in vitro, 2) the responsiveness of the GH cells to hydrocortisone (HC) and hypothalamic GH-releasing hormone (GHRH), 3) the cytoarchitecture of GH cells, and 4) their intracellular hormone content. Evidence for recovery of some, but not all, of these changes during a 6-day postflight test period was obtained. Finally, the data show the influence of paracrine interactions between the heterogeneous cell types on cell function as they experience low gravity.

A companion report describes changes in PRL cells that also occurred during this same experiment (18).

MATERIALS AND METHODS

Animals and Tissue Processing Before Flight

Animal care and use for this experiment, which was flown on the Space Shuttle in 1992 (STS 46), were approved by Institutional Animal Care and Use Committees at both The Pennsylvania State University and the National Aeronautics and Space Administration (NASA) Ames Research Center and conformed to National Institutes of Health guidelines. Nineteen hours before launch, 100 specific pathogen-free Sprague-Dawley male rats (200-220 g; Harlan Sprague Dawley, Frederick, MD) were killed by decapitation and their entire anterior pituitary glands or the dorsal and ventral regions were dissociated into single-cell suspensions with a trypsinization technique that routinely yields $2-2.5 \times 10^6$ cells from each gland (17).

Cells (2.0×10^5 in 200 μ l) from one of five different experimental groups (Fig. 1) were added to a 4.4-ml capacity borosilicate glass vial (Wheaton) containing 4.0 ml of culture medium [either modified Eagle's minimal essential medium (α MEM) containing 5% calf serum, 0.2% NaHCO₃, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.4), and antibiotics; or α MEM containing insulin, transferrin, and selenium (Collaborative Biomedical) buffered with 25 mM HEPES (pH 7.4) and antibiotics] (28, 33). Some of these media were supplemented with 1 μ M HC. The cell groups included 1) the entire population of different hormone-producing cell types (mixed), 2) separated cells of low density [1.040-1.071 g/cm³; (*band 1*)], 3) separated cells of high density [1.071-1.085 g/cm³; (*band 2*)], 4) mixed cells prepared from the dorsal part of the gland, and 5) mixed cells from the ventral part of the gland. A dorsal and ventral section from each gland was made with a Smith-Farquhar tissue chopper. Each gland was positioned on the stage of the device so that a single slice would reproducibly yield two sections of \sim 6,000 μ m each. The method used to separate GH cells into two subpopulations involved layering 10^7 dispersed

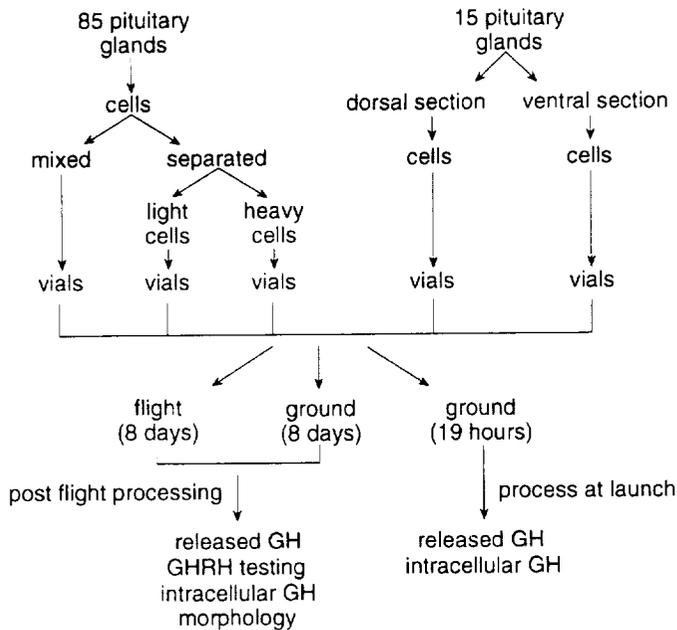


Fig. 1. Experimental design. GH, growth hormone; GHRH, GH-releasing hormone. See MATERIALS AND METHODS for details.

cells (1 ml) over discontinuous density gradients of bovine serum albumin (29). *Layer 1* had a density of 1.071 g/cm³, whereas *layer 2* had a density of 1.085 g/cm³. After centrifugation (2,000 rpm for 30 min at 4°C), cells were collected from the two layering interfaces. Those of densities < 1.071 g/cm³ (*band 1*) have GH cells with relatively few cytoplasmic GH-containing 0.3- μ m secretory granules, whereas those of densities > 1.071 g/cm³ (*band 2*) are laden with these particles (29). The average recovery of cells from the gradient was 86% ($n = 7$ preflight trials), and the GH cell percentages [determined by flow cytometry (15)] in nine trials were 23 ± 3 and $52 \pm 4\%$ for *band 1* and *band 2* cells, respectively. Other preliminary data indicated that *band 2* GH cells contained 100 pg GH/cell, whereas *band 1* cells contained about one-half of that amount. The earlier report of Snyder et al. (29) showed that the addition of 1 μ M HC to serum-containing medium markedly increased the intracellular GH of cultured pituitary cells but had relatively little effect on hormone release; preliminary experiments with 1 μ M HC in the closed-vial system confirmed this result.

There were six sealed cell-containing vials for each of the five experimental groups; the total number of vials exposed to microgravity was 165. Three identical sets of 165 cell-containing vials were kept at 37°C between the time of preparation (launch minus 19 h) until 12 h before landing when they were required to be kept at 22°C. One of the 165 vial sets served as the synchronous ground control; it was kept in the laboratory at the Kennedy Space Center (FL) under conditions identical to those in space during the 8-day flight. The other vial set was processed at launch to determine how much GH was released from the cells into the medium during the 19 h between the end of cell preparation and the launch.

Postflight Cell Culture and GHRH Testing

Postflight processing began after transport to the testing facility (7 h). Some of the cell-containing vials ($n = 3$ vials/flight and 3 vials/ground) were replaced with fresh media, and postflight culture continued in the same sealed vials for an additional 6 days with an intervening medium

change on *day 3*. Cells in other vials from mixed, *band 1*, and *band 2* flight and ground groups (only those in serum-containing medium without HC) were tested for their responsiveness to a synthetic hypothalamic GHRH (2×10^{-9} M; Peninsula Laboratories). This testing was done by 13 successive additions and replacements of fresh medium (1 ml of α MEM + 5% calf serum each time) to each cell-containing vial ($n = 3$ vials/treatment group) at 15-min intervals. Only the fourth and ninth medium changes contained either 2×10^{-9} M GHRH prepared in phosphate-buffered saline (1 μ l) or vehicle alone.

GH Assays

Concentrations of immunoreactive GH (iGH) released from the cells into the culture media were determined by an enzyme immunoassay (8). The polyclonal antiserum to GH has a cross-reactivity of <0.3% to PRL at the final dilution (1:80,000) used in the assay; each sample was analyzed in duplicate at two dilutions, and the results are expressed relative to a rat GH standard preparation (B-11) kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD) and the National Hormone and Pituitary Program (University of Maryland School of Medicine, Baltimore). Intracellular GH was extracted from the cells by overnight incubation in 0.01 N NaHCO₃ (4°C), followed by centrifugation (1,000 g for 30 min) to remove particulate material. Under these conditions, the supernatant fraction contains >90% of the extractable GH (9).

Concentrations of biologically active GH (bGH) in the culture media and extracts were determined exactly according to the tibial-line bioassay procedure of Greenspan et al. (10). Approximately 2,000 hypophysectomized female rats, 26 days old at surgery, were used to assay samples with a four-point assay procedure (i.e., four rats/dose at two doses). The assay's end point measures increases in tibial epiphyseal plate widths after four daily injections of hormone; it has a sensitivity of 1 μ g and is specific for GH. Responses were compared with a bovine GH standard (1.5 U/mg) calibrated against a USP standard; they are expressed in terms of an in-house preparation of rat GH (3.0 IU/mg).

High-Performance Liquid Chromatography (HPLC)

To compare apparent molecular weights of GH released from cells in microgravity vs. unit gravity, 1-ml samples of serum-containing media (± 1 μ M HC) from the mixed, *band 1*, and *band 2* flight samples ($n = 3$ samples/group) or their corresponding ground control samples were lyophilized and reconstituted in 500 μ l of 0.1 M potassium phosphate buffer containing 0.05 M NaCl, pH 7.8. Each sample was applied to a sizing column of Protein-Pak 300 SW (7.8 mm \times 300 mm; Waters, Milford, MA) equilibrated with the same buffer. The column flow rate was 0.3 ml/min, and 0.6-ml fractions were collected. The column was calibrated with blue dextran (mol wt 2,000,000), β -amylase (mol wt 200,000), bovine serum albumin (mol wt 66,000), carbonic anhydrase (mol wt 29,000), and ribonuclease (mol wt 13,683).

Morphology

In some cases, cells were removed from the vials by trypsinization (19) and prepared for immunocytochemistry or flow cytometry. Immunocytochemistry was used to identify GH cells in preparations that were attached to poly-L-lysine-coated coverslips (15). Briefly, this involved fixation in Zamboni's fluid followed by membrane permeabilization with 0.4% Triton X-100, incubation in GH antiserum (1:10,000) for 36 h,

incubation in horseradish peroxidase antiserum (1:500), and staining with diaminobenzidine. These preparations were used for digital analysis of the cytoplasmic area occupied by GH. This procedure and equipment were identical to those used previously (16); it generates a digitized image of the stained cell, colorized according to intensities within the image device's 256 gray levels to quantitate the area of GH staining. As before, care was taken to maintain constant light settings, condenser height, and aperture size in addition to normalizing variations in light with a light meter placed at the level of the camera. In some cases, there were also sufficient cells to do flow cytometric analysis of GH-stained cells in suspension (15). Cell fixation was in phosphate-buffered saline-azide-buffered 4% Formalin followed by membrane permeabilization with 0.4% Triton X-100, incubation in GH antiserum (1:10,000) overnight, incubation in fluorescein isothiocyanate-conjugated antiserum, counterstaining with propidium iodide, and analysis by flow cytometry (Epics model 753). Parameters evaluated on 30,000 cells/sample were 1) GH cell percentage, 2) marker index (the ratio of the voltages of stained to unstained cells; an index of the "brightness" of fluorescence staining), 3) forward-angle light scatter (FALS; an indicator of cell size), and 4) perpendicular light scatter (PLS; an indicator of the content of cytoplasmic hormone-containing secretory granules) (15). The application of the flow cytometer to evaluate these parameters for rat pituitary cells, as well as the experimental data that establish their biological significance, has been documented (25).

Closed-Cell Culture System: Validation

Because it was not possible to do this experiment on the Space Shuttle with conventional cell culture hardware, a simple passive system was developed. Release of iGH from cells in the closed-vial system over a 9-day period at 37°C was linearly related ($r^2 = 0.992$) to cell number over the range of 10^3 to 8×10^5 cells seeded (9 trials). The amount of iGH synthesized by 2×10^5 cells during the 9 days (i.e., the amount recovered in media plus cells minus the amount initially seeded) ranged between 15 and 20 μg . A majority of synthesized GH was released into the culture medium. Net GH synthesis in the closed-vial system was not significantly different from that measured in primary rat pituitary cell cultures that are routinely maintained on conventional plasticware (Linbro tissue culture plates) that allows free gas exchange (95% air-5% CO_2) (33). It was also not significantly different from that measured in cells cultured on Linbro plates that were sealed to prevent gas exchange (data not shown).

Several formulations of culture media were tested to determine optimal GH synthesis in the closed system; these included Medium 199, αMEM , addition of 0.2% NaHCO_3 , and calf or horse serum (either 5 or 10%). In addition, we compared GH synthesis levels in cells cultured in rat serum with those cultured in calf or horse serum. The rank order was determined to be rat serum > calf serum > horse serum. Cells in rat serum synthesized 2.3 ± 0.4 times as much hormone as those in calf serum ($n = 14$ experiments);

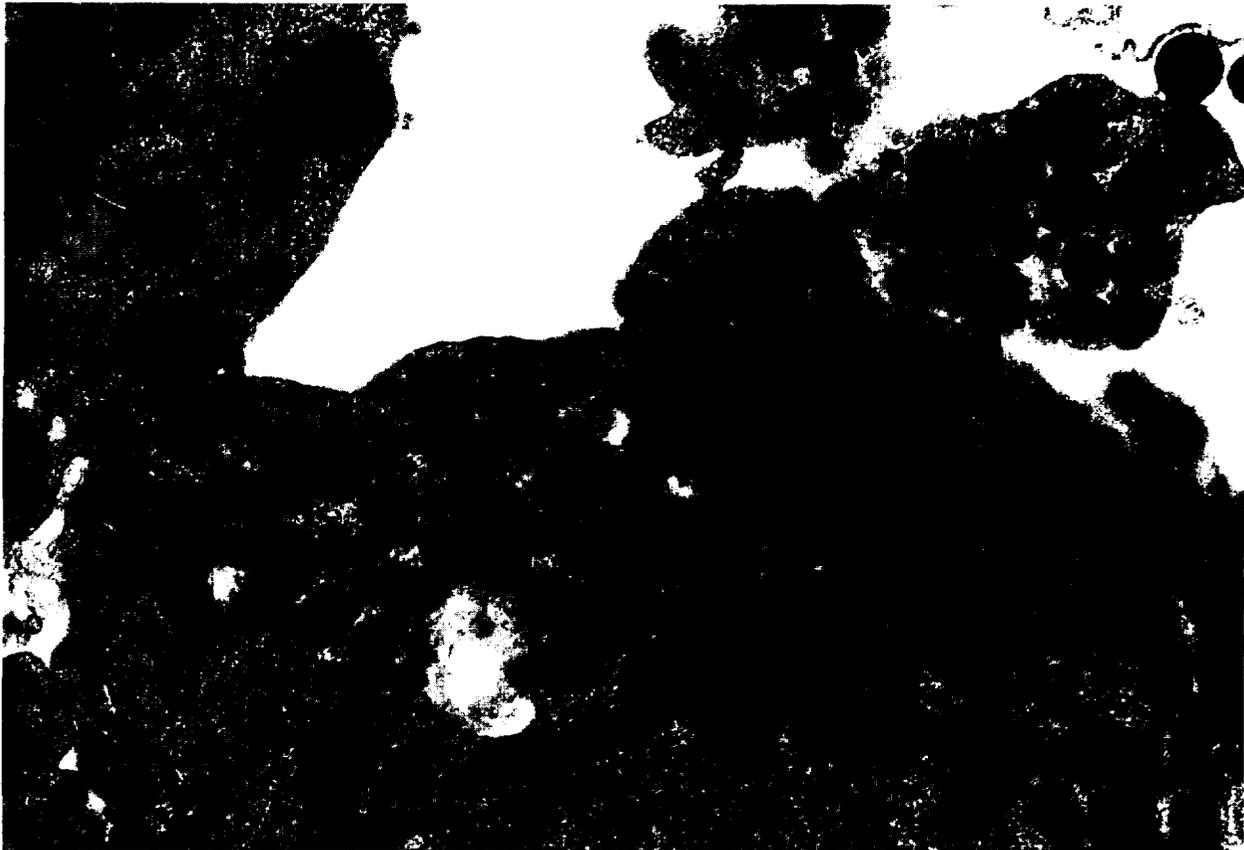


Fig. 2. Electron micrograph of a GH and prolactin cell maintained in a closed glass vial for 9 days at 37°C before trypsinization and preparation for electron microscopy. Cell ultrastructure compares favorably with cells maintained under more usual culture conditions. Magnification, $\times 25,000$.

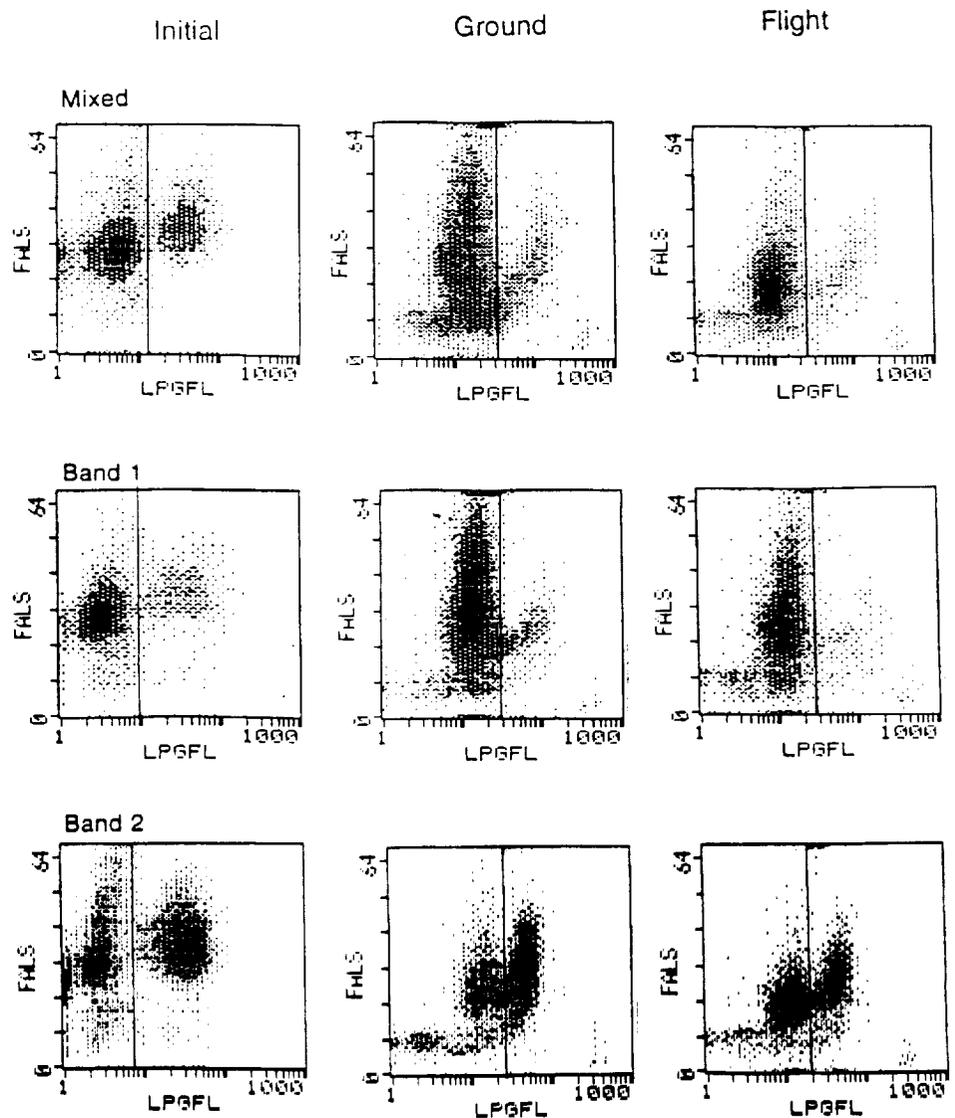


Fig. 3. Flow cytometric histograms of pituitary cells contained in the mixed (top row), band 1 (middle row), and band 2 (bottom row) samples before seeding into glass vials (initial; left column) and after 9 days of culture under unit gravity (ground; middle column) or microgravity (flight; right column) conditions. Log peak green fluorescence (LPGFL) staining is specific for GH cells and delineates stained [identified as cells falling to right of vertical gate (vertical line) in each panel] from unstained cells that include other hormone-producing cell types gated from debris on basis of propidium iodide nuclear stain. LPGFL is on a log scale, whereas forward-angle light scatter (FALS), a measure of cell size, is linear. Each pattern represents a count of 30,000 cells.

however, it was not selected for use in the flight experiment because rat serum supplies were limiting. With 2×10^5 cells in closed vials for 9 days, the pH is maintained within 0.1 unit of its initial value (pH 7.4).

After 9 days, the cells showed a typical ultrastructure including intact membranes around the secretion granules (Fig. 2). Other data showed that $53 \pm 3\%$ ($n = 14$ trials) of the cells originally seeded could be recovered by trypsinization after 9 days; the percentage of recovered GH cells ($29 \pm 3\%$; $n = 11$ experiments) compared favorably with input percentage, demonstrating that preferential loss of GH cells did not

occur during trypsinization. Amounts of GH released from every 1,000 GH cells seeded during 9 days in culture was 336 ± 9 ng ($n = 15$ experiments).

Microgravity Cell Culture: Special Considerations

It was necessary to establish that the vibration forces associated with the Shuttle launch and recovery would have no deleterious effect on cell attachment, structure, or function. We found $>97\%$ of the cells seeded into the vial routinely attached to the vial bottom within 18 h; furthermore, they

Table 1. Effect of microgravity on cell parameters measured by flow cytometry

	GH Cells, %			Total Cell Population				GH Cell Population					
	Before flight	After flight		FALS		PLS		Marker index		FALS		PLS	
		Flight	Ground	Flight	Ground	Flight	Ground	Flight	Ground	Flight	Ground	Flight	Ground
Mixed	32	12	11	21	25	21	33	5.2	6.1	23	24	57	66
Band 1	22	9	11	27	30	29	37	4.4	3.9	20	23	34	49
Band 2	57	47	62	23	27	42	58	2.1	2.2	25	29	75	84

Values represent peak channels of size [forward-angle light scatter (FALS)] and cytoplasmic granularity [perpendicular light scatter (PLS)] and ratio of voltages of stained to unstained cells (marker index). Data were collected from 30,000 cells/sample. GH, growth hormone.

remained attached after vibration tests were conducted at the NASA Ames Research Center that were greater than those typically experienced on the Shuttle. Furthermore, shaking had no effect on subsequent release of iGH during a 9-day culture test.

Because there was a 0.2-ml air bubble at the top of each vial and because bubble behavior can be unpredictable in space, we were concerned that it could become dislodged in flight, come to rest directly over the cell layer, and result in cell dehydration. To discourage this possibility, each vial was rimmed internally (upper one-third) with a surface active agent (Prosil 28, Thomas Scientific); furthermore, one time each day, an astronaut rotated the vial container five times in a period of 10 s.

We also conducted a trial aboard the KC-135 airplane to study bubble behavior in the glass vial during the 20–30 s of microgravity achieved in parabolic flight and verified that the air bubble tended to remain in the upper one-third of the filled vial.

Data Presentation

We determined that the amount of GH released during the 19 h before the Shuttle launch (Fig. 1) was <1% of the total

GH released over the entire 8-day culture period; these data are therefore not included in RESULTS.

Approximately one-half of the cell culture vials used a chemically defined serum-free medium that was supplemented with insulin, transferrin, and selenium. This formulation was used to eliminate possible effects of unknown serum factors on GH cells. In every case, the trends in GH release between various treatment groups were identical to those using serum-containing media except that the actual GH levels were consistently lower. We have therefore chosen not to present these results in this report.

Two automatic temperature recording devices, developed at the NASA Ames Research Center, were secured in the sealed vial containers to continuously monitor ambient temperature around the vial sets during spaceflight and in the laboratory at the Kennedy Space Center. The sensitivity of the temperature recorder was $\pm 0.4^\circ\text{C}$. The recordings indicated that the control cells experienced temperatures ranging between 37.3 and 37.6°C for the 9-day experiment while the flight cells experienced temperatures between 38.4 and 38.8°C for the same period. Further analysis indicated that the average temperature difference was 1.2°C between flight and ground. The entire flight experiment was therefore repeated

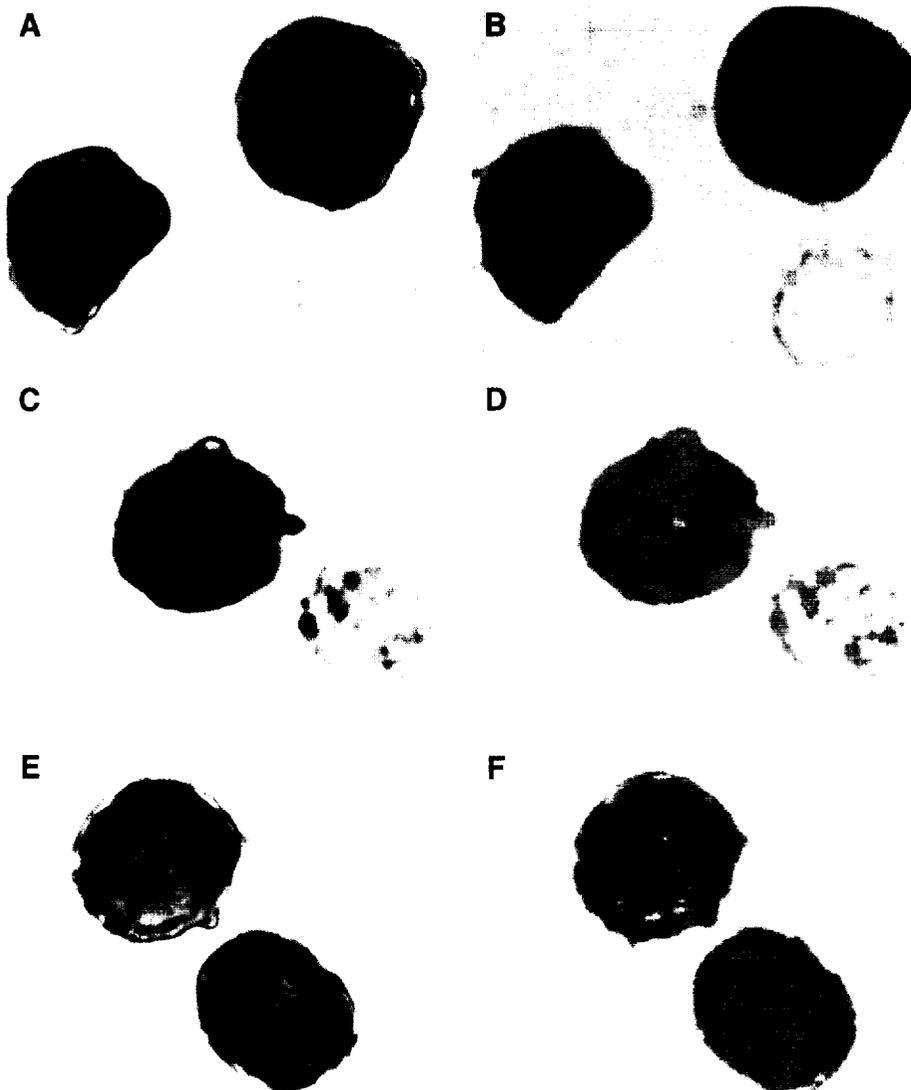


Fig. 4. Examples of GH cells stained immunocytochemically (A, C, and E) and their digitized color images (B, D, and F) used to quantitate their cytoplasmic areas occupied by GH. Examples of GH cells in mixed (A and B), band 1 (C and D), and band 2 (E and F) cells are shown. Magnification, $\times 1,500$.

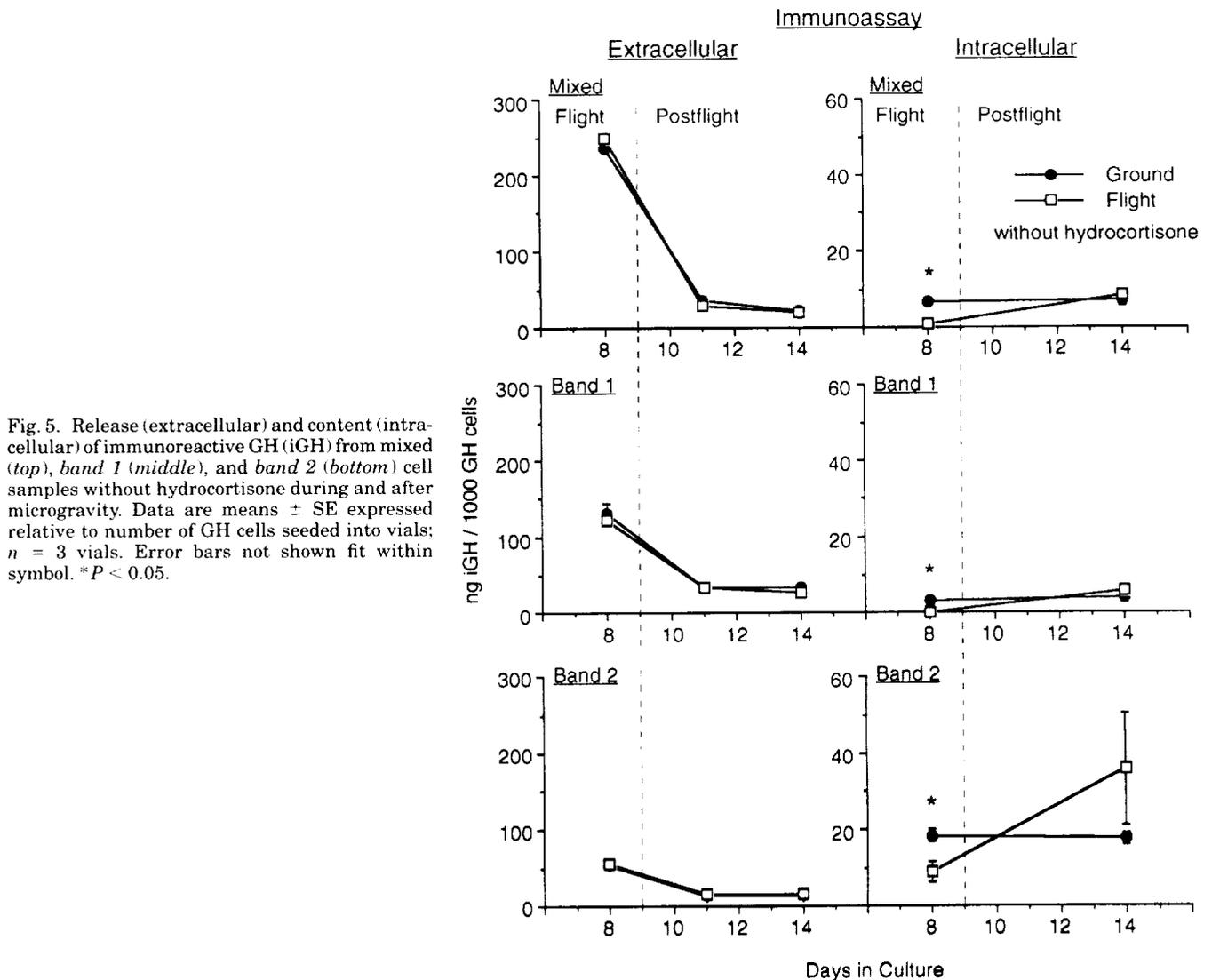


Fig. 5. Release (extracellular) and content (intracellular) of immunoreactive GH (iGH) from mixed (top), band 1 (middle), and band 2 (bottom) cell samples without hydrocortisone during and after microgravity. Data are means \pm SE expressed relative to number of GH cells seeded into vials; $n = 3$ vials. Error bars not shown fit within symbol. * $P < 0.05$.

6 mo later in the laboratory at The Pennsylvania State University where cells in the closed vials were kept at either 39 (to mimic flight temperatures) or 37°C for 9 days. A statistical comparison of the data between these two experiments with a logarithmic transformation methodology further showed that the changes in the flight experiment could not be explained on the basis of a temperature differential. We have therefore chosen to present only the synchronous ground control data in this report.

Experimental constraints within this spaceflight investigation resulted in low n sizes. Accordingly, multiple α -level-corrected independent t -tests were used to analyze these data. This, in turn, permitted use of an inferential technique to highlight differences as well as strong trends. Significance was maintained at $P \leq 0.05$.

RESULTS

GH Cell Morphology

The viability of all cell samples both before and after flight was $>90\%$. At the end of the experiment, the pH of the culture medium was 7.4. Microscopy showed that all cells had remained attached to the bottom of each

vial. The general morphology of the cells after spaceflight, while still attached, was unremarkable and similar to the cultured ground control samples; steroid treatment had no obvious effect on cell morphology.

Flow cytometric histograms of GH-specific log peak green fluorescence vs. FALS signals show that several changes had occurred between the time of initial cell preparation and recovery from the vials after the 10-day culture. These included 1) broader FALS patterns in unstained cells (mixed and band 1 samples) after culture than before culture, 2) decreased FALS signals of unstained cells in band 2 during the ground and flight cell culture periods, and 3) maintenance of the FALS pattern of GH cells in the band 2 sample during the entire experiment but 4) a lack of maintenance of the FALS pattern of GH cells in the mixed and band 1 samples over the 10-day period (Fig. 3). Table 1 shows data derived from these histograms that compare the effects of flight on 1) the GH cell frequency, 2) the mean FALS-to-PLS peak channel of the total and GH cell populations, and 3) the marker index of the GH

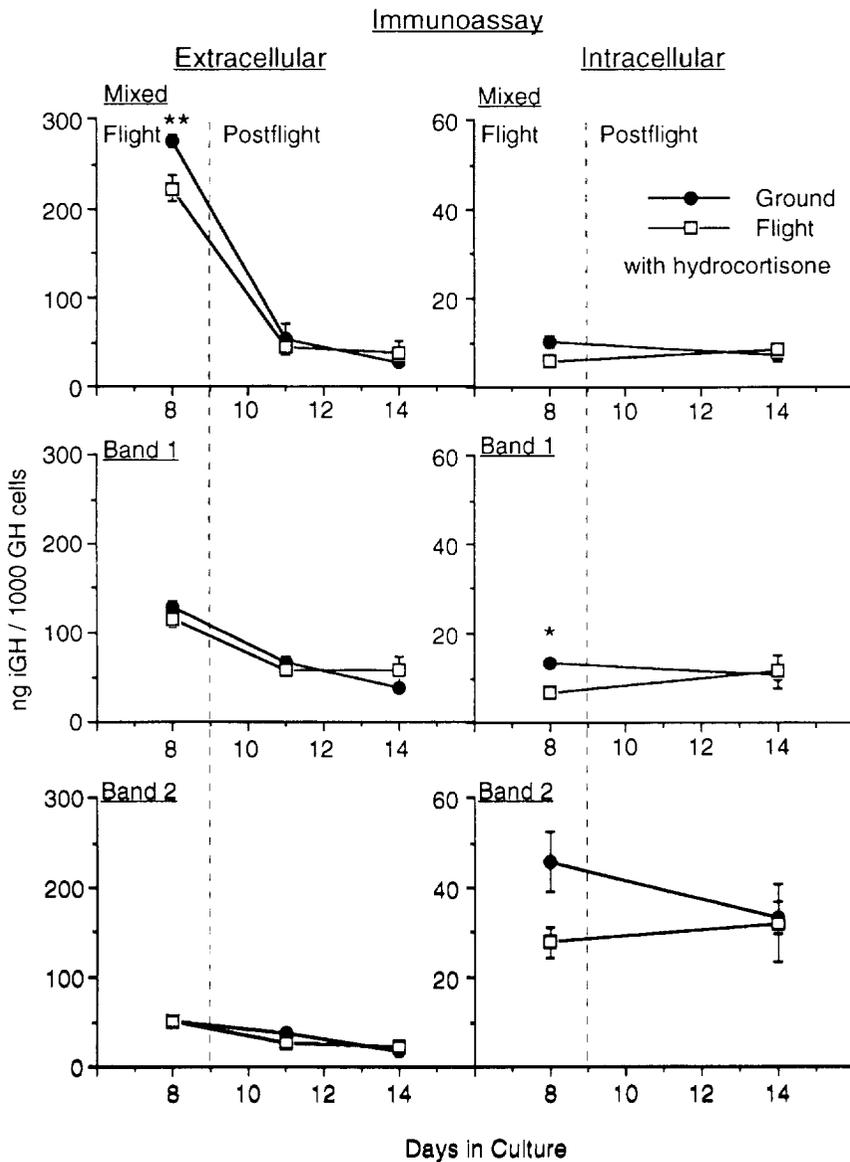


Fig. 6. Release and content of iGH from mixed (top), band 1 (middle), and band 2 (bottom) cell samples maintained in hydrocortisone-containing medium during and after microgravity. Data are means \pm SE expressed relative to number of GH cells seeded into vials; $n = 3$ vials. Error bars not shown fit within symbol. * $P < 0.05$. ** $P < 0.01$.

cells. Because PLS signals are on a logarithmic scale (not shown in Fig. 3), the lower mean channel values of all flight cells are considered significant. On the other hand, differences in the FALS and marker index were small (linear scale); we conclude that neither the fluorescence intensity of GH staining nor the size of the pituitary cells was affected by spaceflight. Determination of GH cell frequency by manual counting (not shown) was consistent with the data in Table 1. We attribute the changes in GH cell FALS patterns after the experiment to the cell culture procedure per se; supporting evidence for this idea can be found in Ref. 15. However, we have no explanation why band 2 cells maintain discrete FALS patterns when they are in isolation but not when they are present as a part of the mixed sample.

Image analysis of individual GH cells from both mixed and band 2 groups after flight indicated that, relative to their ground control samples, there were statistically significant increases in their cytoplasmic

areas that contained hormone. This was not true in band 1 cells. GH percent area occupancies measured on 200 cells/sample were $40 \pm 3\%$ for ground vs. $53 \pm 2\%$ for flight mixed samples ($P < 0.001$), $39 \pm 3\%$ for ground vs. $37 \pm 2\%$ for flight band 1 samples (not significant), and $27 \pm 2\%$ for ground vs. $40 \pm 2\%$ for flight band 2 samples ($P < 0.001$). Examples of immunocytochemically stained GH cells and their digitized images used to collect these data are shown in Fig. 4.

Extracellular and Intracellular GH During and After Flight

iGH \pm HC. Relative to ground control samples, microgravity had no effect on the release of iGH during the 8 days in space nor during the subsequent 6-day postflight culture period. Immediately on landing, the intracellular contents of iGH were significantly lower than in the ground control samples; however, this difference was not maintained after the postflight

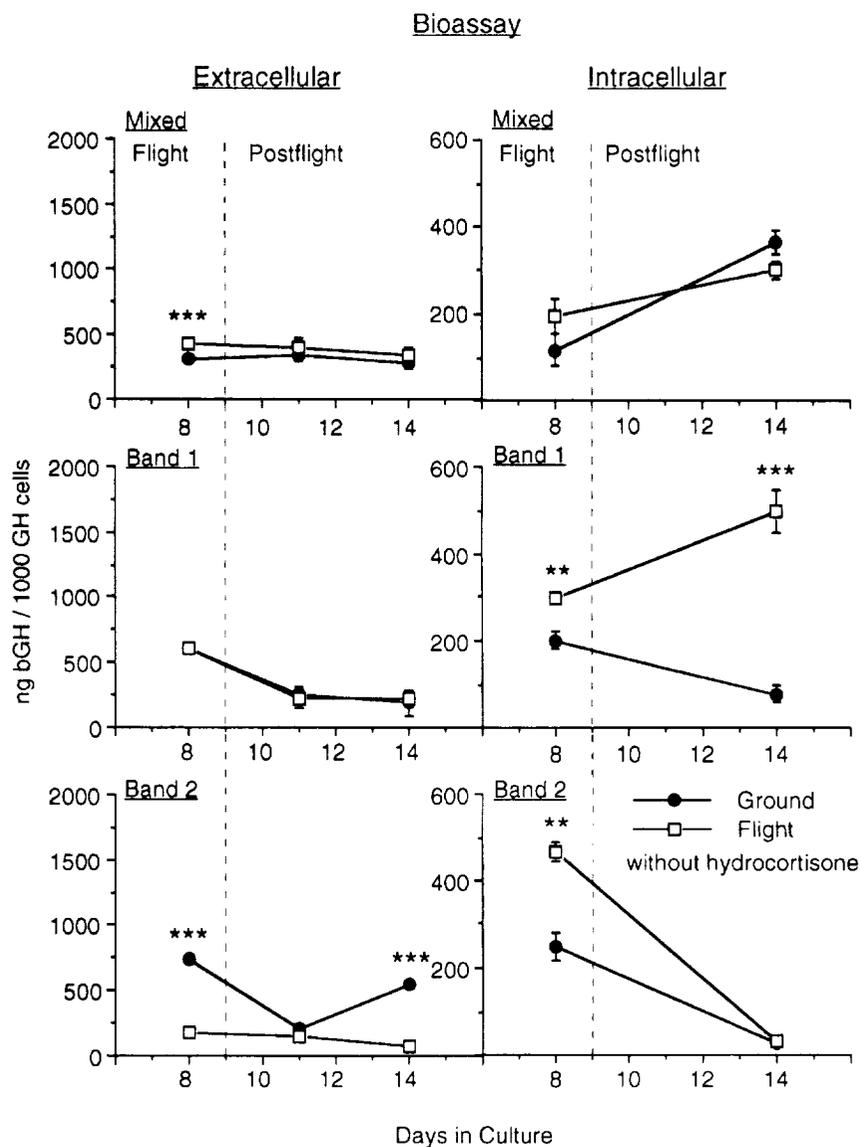


Fig. 7. Release and content of bioactive GH (bGH) from mixed (top), band 1 (middle), and band 2 (bottom) cell samples without hydrocortisone during and after microgravity. Data are means \pm SE expressed relative to number of GH cells seeded into vials; $n = 3$ vials. Error bars not shown fit within symbol. ** $P < 0.01$. *** $P < 0.001$.

culture (Fig. 5). The addition of HC to the culture medium marginally increased iGH release from the mixed ground cell sample and caused a twofold increase in the intracellular iGH in band 2 cells of both ground and flight groups; the relative difference in intracellular iGH between these groups measured in the absence of HC (Fig. 5) tended to continue in the presence of HC (mixed, $P < 0.055$; band 2, $P < 0.057$; Fig. 6).

bGH \pm HC. Relative to the ground control samples, major changes in the release of bGH, especially in HC-containing media, were found (cf. Figs. 7 vs. 8). In Earth's gravity, more bGH was released from band 2 cells than its flight counterpart; addition of HC reversed the effect. In space, the opposite was true; i.e., HC stimulated bGH release from band 2 cells back to the levels of non-HC-treated earthbound band 2 cells. This response continued into the postflight period.

Band 1 cells from both ground and flight samples released considerably more (two to three times) bGH in

the presence of HC than in its absence; this was not true for GH cells in the band 2 samples. Effects of microgravity on intracellular GH were complex. In general, flight cells cultured without HC often contained significantly less bGH, but all cell groups cultured in the presence of HC in microgravity consistently contained significantly less bGH.

HPLC analysis of extracellular iGH. Culture media taken from cells after 8 days in microgravity were found to contain iGH molecules of widely different apparent molecular weights. In general, neither microgravity nor steroid had any marked effect on the size distribution profile of the hormone (Fig. 9). The most important change was the tendency for flight samples to contain more GH of higher apparent molecular weight. To make statistical comparisons between the amounts of iGH contained in different molecular-weight regions of these chromatograms as a function of gravity level, cell sample, and steroid, we compared the sums of iGH contained in three different molecular-

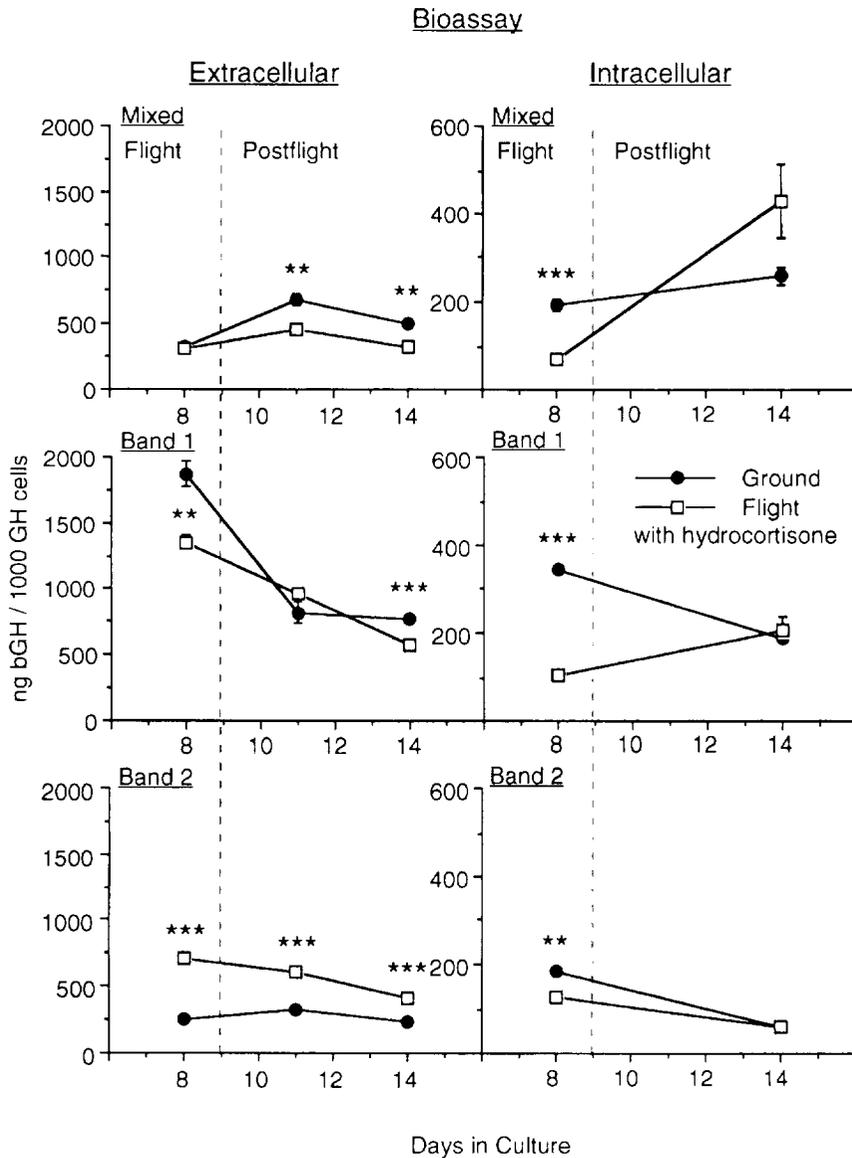


Fig. 8. Release and content of bGH from mixed (top), band 1 (middle), and band 2 (bottom) cell samples maintained in hydrocortisone-containing medium during and after microgravity. Data are means \pm SE expressed relative to number of GH cells seeded into vials; $n = 3$ vials. Error bars not shown fit within symbol. ** $P < 0.01$. *** $P < 0.001$.

weight regions (high, 9.9×10^5 to 2.4×10^5 ; medium, 2.4×10^5 to 5.9×10^4 ; low, 5.9×10^4 to 1×10^4). The only statistically significant effect ($P \leq 0.05$) was the reduced amount of iGH recovered from the low-molecular-weight regions of the mixed HC-treated cell flight samples (Fig. 9D). Strong tendencies toward differences between flight and ground were noted in the following samples: 1) mixed, without HC, high molecular weight: flight $>$ ground ($P < 0.08$); 2) band 1, with HC, high molecular weight: flight $>$ ground ($P < 0.07$); and 3) band 2, without HC, medium molecular weight: flight $>$ ground ($P < 0.10$). In general, the total amounts of iGH recovered after HPLC from flight and ground samples were within 10% of each other. Relative to separated cells, a greater fraction of iGH was of higher apparent molecular weight when the total cell population was used. The reason for this difference is not known.

Response to GHRH. After 8 days in low gravity, cell responses (iGH release) to 10^{-9} M GHRH challenge

were only moderate to nonexistent, whereas the responses of ground-based mixed and band 1 cells were significant (Fig. 10). The lower sensitivity of the GH bioassay required combining five 15-min fractions (including the GHRH pulse) before assay. These results also showed 1) nonresponsiveness of mixed cells after flight (Fig. 11), 2) apparent increased sensitivity of band 1 cells to GHRH (Fig. 12), and 3) weak to nonexistent responses of band 2 cells (Fig. 13).

Substrate. Three vials pretreated with Matrigel (a commercially available mixture of basement membrane macromolecules) and then loaded with mixed cells were also exposed to low gravity. This treatment had no effect on the release of either iGH or bGH from cells regardless of treatment group, i.e., flight, ground, and postflight (data not shown). Band 1 and 2 cells were not tested.

Cell location. Flight had no effect on the release of iGH from mixed cells prepared from either dorsal or ventral regions of the pituitary gland [dorsal, 367 ± 22

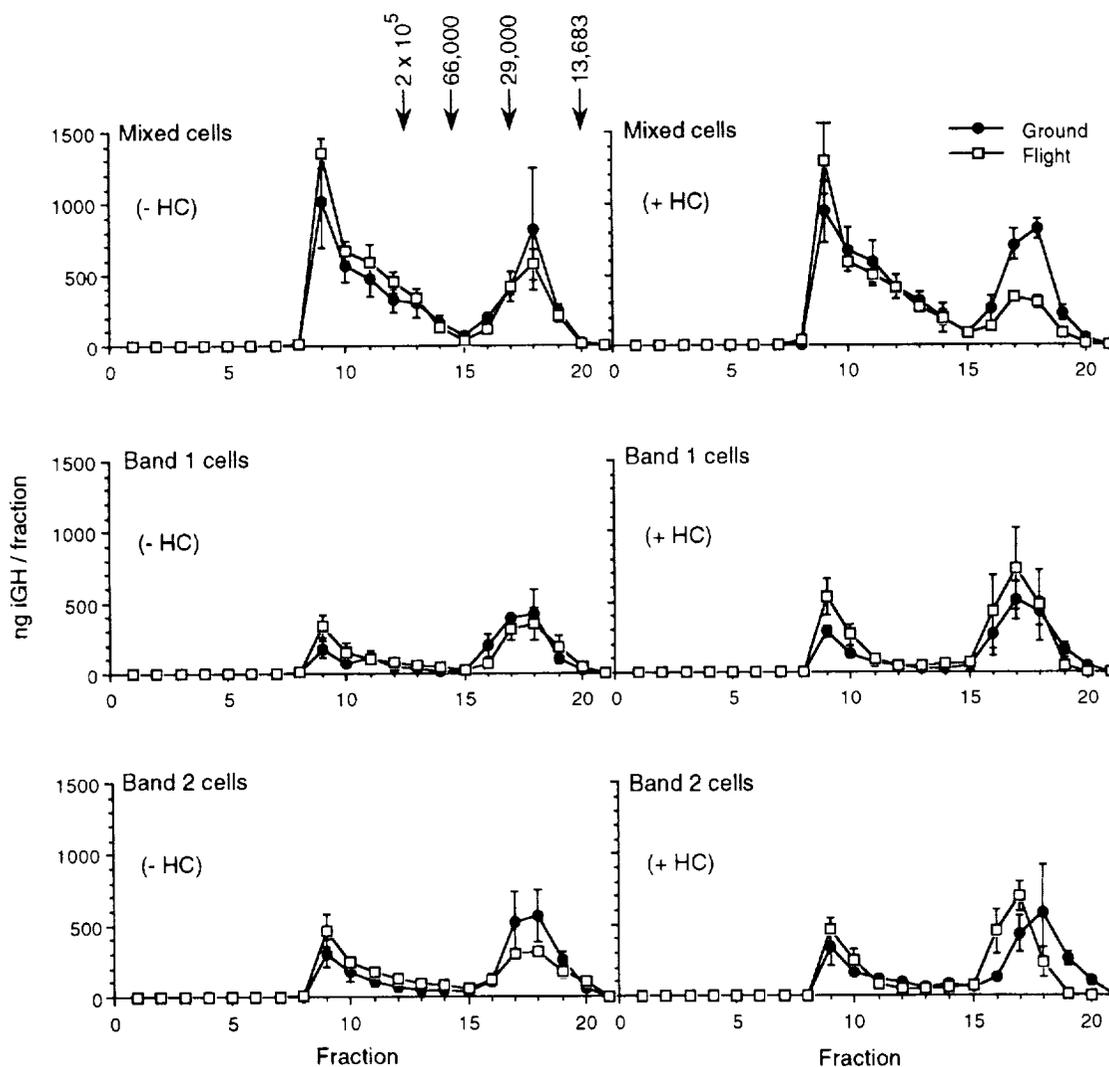


Fig. 9. Fractionation of culture media without (-HC) and with hydrocortisone (+HC) from spaceflown and ground control cells by gel exclusion high-performance liquid chromatography ($n = 3$ separate sample media/group). Elution position of standard molecular-weight markers (see MATERIALS AND METHODS) are shown in upper left panel. For ease of comparison of these data, see Table 2.

(flight) and 347 ± 14 ng/1,000 GH cells (ground); ventral, 150 ± 7 (flight) and 169 ± 7 ng/1,000 GH cells (ground)]. However, release of bGH from ventral flight cells was reduced by one-half [dorsal, 116 ± 4 (flight) and 108 ± 15 ng/1,000 GH cells (ground); ventral, 240 ± 10 (flight) and 545 ± 19 ng/1,000 GH cells (ground)]; $P < 0.001$].

Intracellular GH variants. Western blots of cell extracts after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions revealed a complex array of iGH forms that were qualitatively similar between flight and ground samples (Fig. 14A). Analysis of these various cell samples by densitometry showed that although differences between flight and ground did not vary within any given region by $>10\%$, the pattern of changes depended on both the sample and presence of HC. For example, flight tended to increase amounts of higher molecular-weight iGH forms in *band 1* cells at the expense of lower molecular-weight forms and HC accen-

tuated that difference (Fig. 14C). However, a similar analysis of iGH from *band 2* flight cells give very different profiles; in this case, HC reversed this pattern (Fig. 14D). Not surprisingly, the mixed cell sample gave a pattern that was different (Fig. 14B). Comparison of the molecular-weight data, i.e., patterns of iGH variants contained inside cells (Fig. 14) vs. those released from cells (Fig. 9), show an obvious correlation between the two data sets. Heterogeneity of molecular forms of GH are known to result from mRNA splicing, posttranslational modifications (e.g., aggregation or glycosylation), and proteolytic cleavage.

DISCUSSION

The primary objective of this cell culture experiment was to find out whether the postflight changes in pituitary GH cells prepared from rats after 7–14 days in space also happened when GH cells themselves were put into space. Within the constraints of the hardware

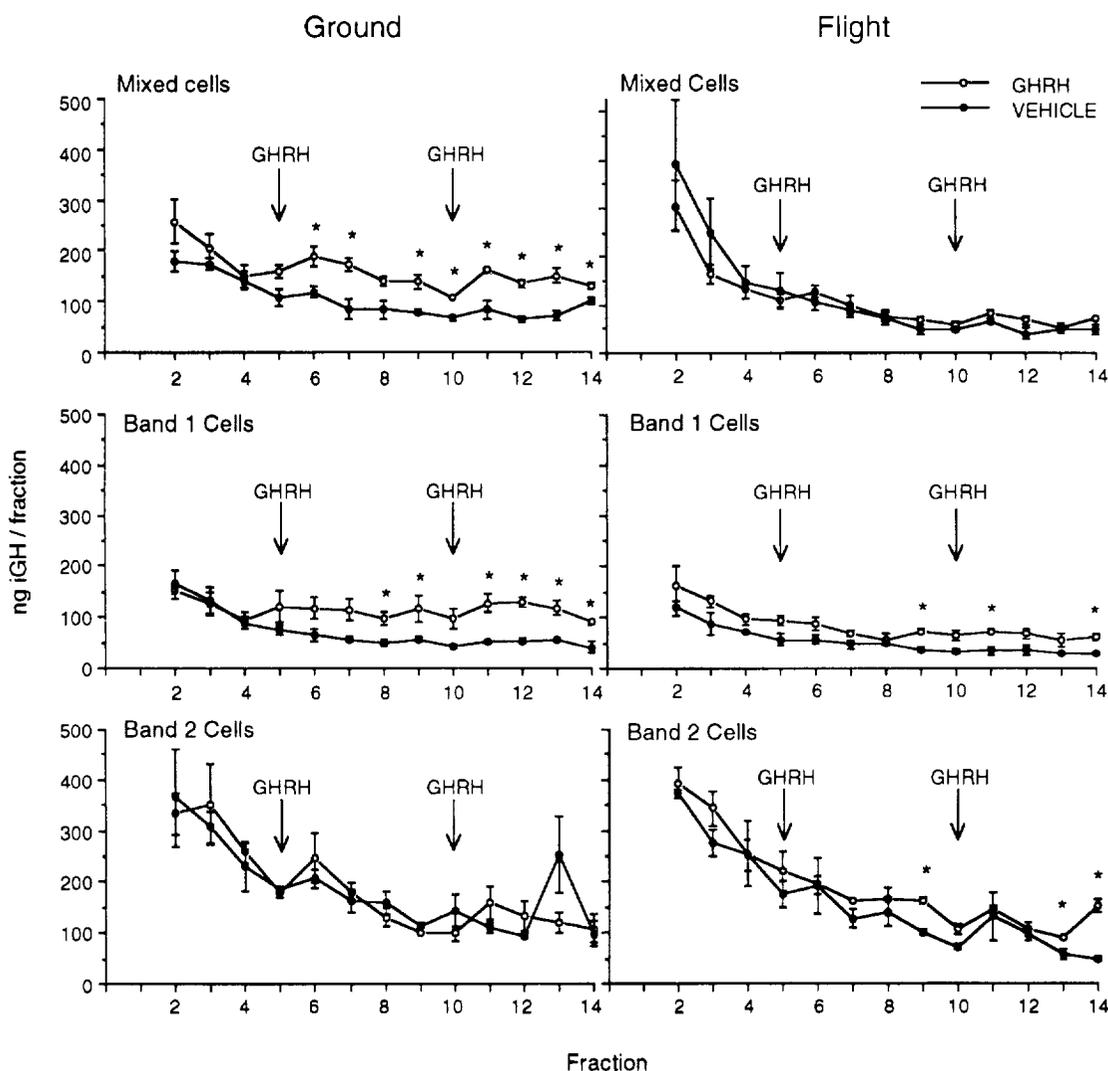


Fig. 10. Responses of pituitary cell samples to two 15-min pulses of 2×10^{-9} M GHRH after culture in closed vials on Earth (left) or in microgravity (right) for 9 days. Media changes occurred once every 15 min, and entire 210-min experiment was done on cells attached to bottom of their original vials. Each data point represents mean \pm SE from 3 vials receiving either peptide (arrows) or phosphate-buffered saline (vehicle). * $P < 0.05$.

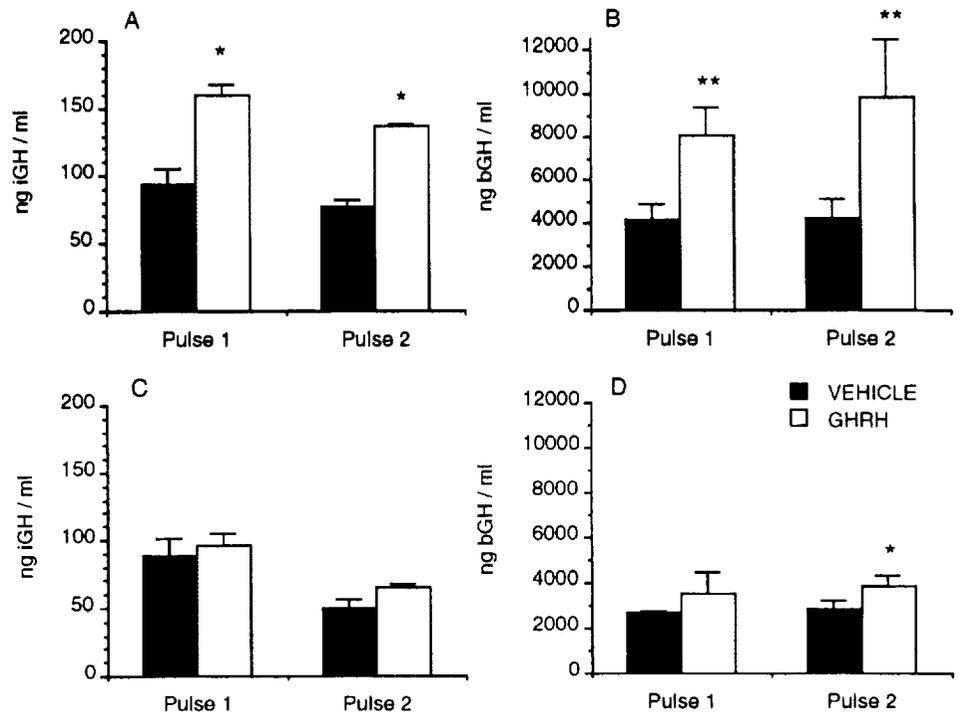
and safety issues imposed by NASA, this objective was largely achieved. Hymer et al. (16) previously reported that certain structural and functional changes in GH cells consistently occurred in spaceflown animals; the present report shows that similar changes also take place in spaceflown GH cells. These include 1) changes in the release of bGH and iGH, 2) changes in the intracellular GH content, and 3) increases in the cytoplasmic area occupied by GH. In addition, this cell culture experiment provides new information regarding microgravity-induced changes in 1) responsiveness of GH cells to adrenal and hypothalamic hormones, 2) paracrine interactions between different hormone-producing cell types, and 3) changes in the apparent mass of some iGH molecules released into the culture medium during flight. A convenient way to summarize the many statistically significant effects in both intracellular and released GH during and after spaceflight, considered within the context of hormone assay, cell type, and culture medium, is given in Fig. 15. This

representation, taken together with other results presented in this paper, enable us to obtain "bottom-line" answers to the questions posed in Table 2.

Our previous spaceflight experiment (16) showed the importance of measuring GH by bioassay in addition to immunoassay. Both assays are specific, but they obviously have different end points. The polyclonal antiserum we use is directed against 22,000 GH. The bioassay is specific; it measures activity of the GH molecule, i.e., its ability to stimulate the growth of long bones of the hypophysectomized female rat (10). Which and how many of the multiple GH variants contained in (and released from) GH cells have activity in this assay is still not fully understood (23). GH molecules released postflight from cells of spaceflown rats consistently show a $\sim 50\%$ suppression in biological activity relative to ground control samples, whereas results by immunoassay tend to be highly variable (16).

The interacting variables of cell type and steroid in the medium clearly dictate the results in terms of GH

Fig. 11. Cumulative release of iGH (A and C) and bGH (B and D) from mixed ground (A and B) and flight (C and D) pituitary cells during GHRH testing. Pulse 1, sum of hormone contained in fractions 5-9. Pulse 2, sum of hormone contained in fractions 10-14. Amounts of iGH released per fraction are shown in Fig. 10; lower sensitivity of GH bioassay required pooling of fractions as indicated. Values are means \pm SE. * $P < 0.05$. ** $P < 0.01$.

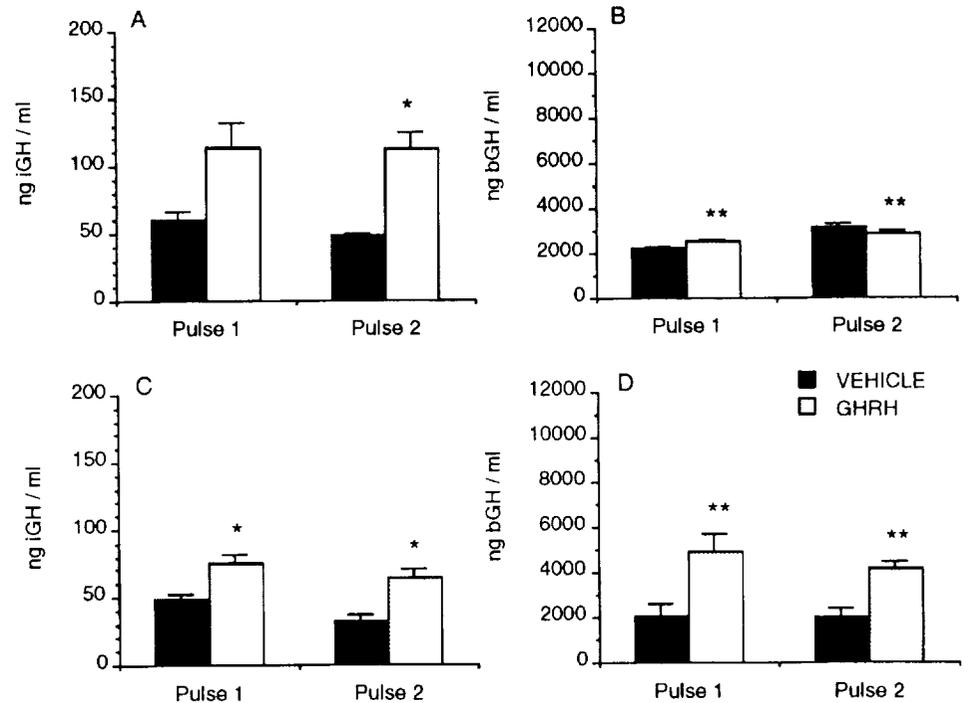


activity. For example, mixed cells release about the same amount of bGH (300-400 ng/1,000 GH cells) regardless of culture medium and gravity level. However, release of bGH from *band 1* cells is two- to threefold higher than from mixed cells in steroid-containing medium; in this GH cell subpopulation, microgravity also appears to play a minor role. On the other hand, the responses of the dense *band 2* GH cells to the variables of gravity and steroid are more interest-

ing. For example, HC suppresses the release of bGH from earthbound dense GH cells during the entire 2-wk culture period, whereas microgravity exposure totally reverses this response. These data not only show that dense GH cells are sensitive to microgravity but also show that their response can be modified by hormones *in vitro*.

Why should dense GH cells, both in the presence and absence of steroid, show such a different behavior in

Fig. 12. Cumulative release of iGH (A and C) and bGH (B and D) from *band 1* ground (A and B) and flight (C and D) pituitary cells during GHRH testing. Pulse 1, sum of hormone contained in fractions 5-9. Pulse 2, sum of hormone contained in fractions 10-14. Amounts of iGH released per fraction are shown in Fig. 10; lower sensitivity of GH bioassay required pooling of fractions as indicated. Values are means \pm SE. * $P < 0.05$. ** $P < 0.01$.



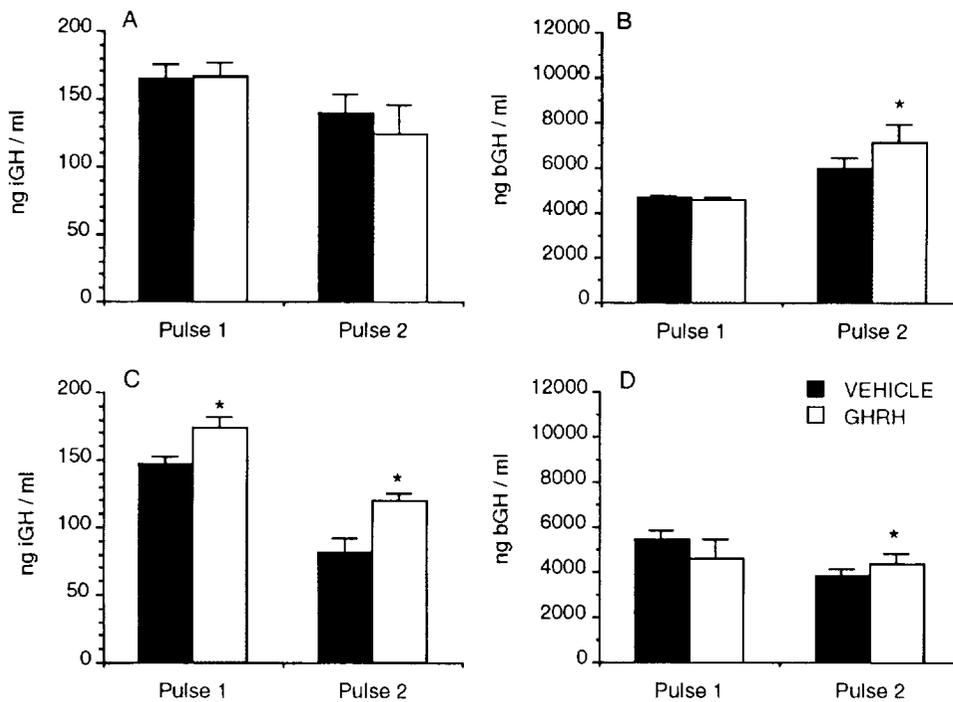


Fig. 13. Cumulative release of iGH (A and C) and bGH (B and D) from *band 2* ground (A and B) and flight (C and D) pituitary cells during GHRH testing. Pulse 1, sum of hormone contained in *fractions 5-9*. Pulse 2, sum of hormone contained in *fractions 10-14*. Amounts of iGH released per fraction are shown in Fig. 10; lower sensitivity of GH bioassay required pooling of fractions as indicated. Values are means \pm SE. * $P < 0.05$.

microgravity? We know that spaceflown GH cells experience an important biophysical change in their cytoplasm, viz., a significantly increased ($\sim 15\%$) area occupied by hormone. This change also happens when cells are prepared from spaceflown rats [two separate flight experiments (16)]. We speculate that secretory granule-microtubule associations and cytoplasmic and/or nuclear steroid receptors in dense GH cells could account for their different behavior. Specifically, all secretory granules are associated with the cytoskeleton via microtubules (27), and the cytoskeleton is often considered to be a primary gravity-sensing structure (19). On Earth, GH cells respond to glucocorticoids via nuclear receptors after the steroid reaches this site via a cytoplasmic-receptor shuttle mechanism believed to involve hormone-heat shock protein complexes (21). We envisage dynamic biophysical-biochemical interactions involving steroid receptors, heat shock proteins, and cytoskeletal-associated elements that would enable the cell to respond to changes in its environment. Thus dense GH cells, "unloaded" in microgravity, might respond to environmental change in a biochemical sense (conformation of the GH molecule) accomplished by way of 1) redistribution of secretory granules and 2) altered activity of molecular chaperones that ultimately result in reduced biological activity of the hormone-secreted molecule. Biophysical changes of the type we envision have been seen in many cells after heat stress (32) and shear stress (3). Because HC exposure stimulated bGH release from *band 2* cells in microgravity (Fig. 8), we predict that GH cytoplasmic area occupancy in dense GH cells would be restored (i.e., decreased) to ground control levels in the presence of steroid. Unfortunately, we did not have sufficient *band 2* cells to test this idea.

The reason(s) why less dense GH cells (*band 1*) releases large amounts of bGH in response to steroid is unknown. Clearly, microgravity appears to play a far less important role in the dynamics of this response. Equally certain, the mechanism(s) by which steroid affects bGH release in *band 1* cells in space must be different from that in *band 2* cells because bGH is significantly suppressed in the former case but significantly elevated in the latter case (Fig. 15).

One striking difference between the results obtained by using immunoassay vs. bioassay was that of the difference in relative amounts of GH released in microgravity from the mixed vs. separated cells. Thus iGH levels were approximately two times greater from the mixed than from the separated cells (200–250 vs. 50–100 ng iGH/1,000 GH cells; Figs. 5 and 6), and steroid made no difference in this pattern. On the other hand, release of bGH from separated GH cell subpopulations was two to six times greater than from the mixed cells (Figs. 7 and 8). It may be that these different results are attributable either to the assay itself, paracrine interactions, or some combination of the two. The polyclonal antiserum we used probably detects many epitopes on the GH molecule, but only a certain percentage of these may actually achieve the proper conformation to confer a biological response in the assay animal. Paracrine interactions between different hormone-producing cell types in the anterior pituitary are known but are difficult to demonstrate conclusively. Although the tibial-line bioassay is known to be specific for GH, it is conceivable that another hormone in the assay sample could interact in the assay animal to give the bone growth response observed. Equally possible, a molecule(s) from dense cells might repress the function of less dense cells (or vice versa) when they

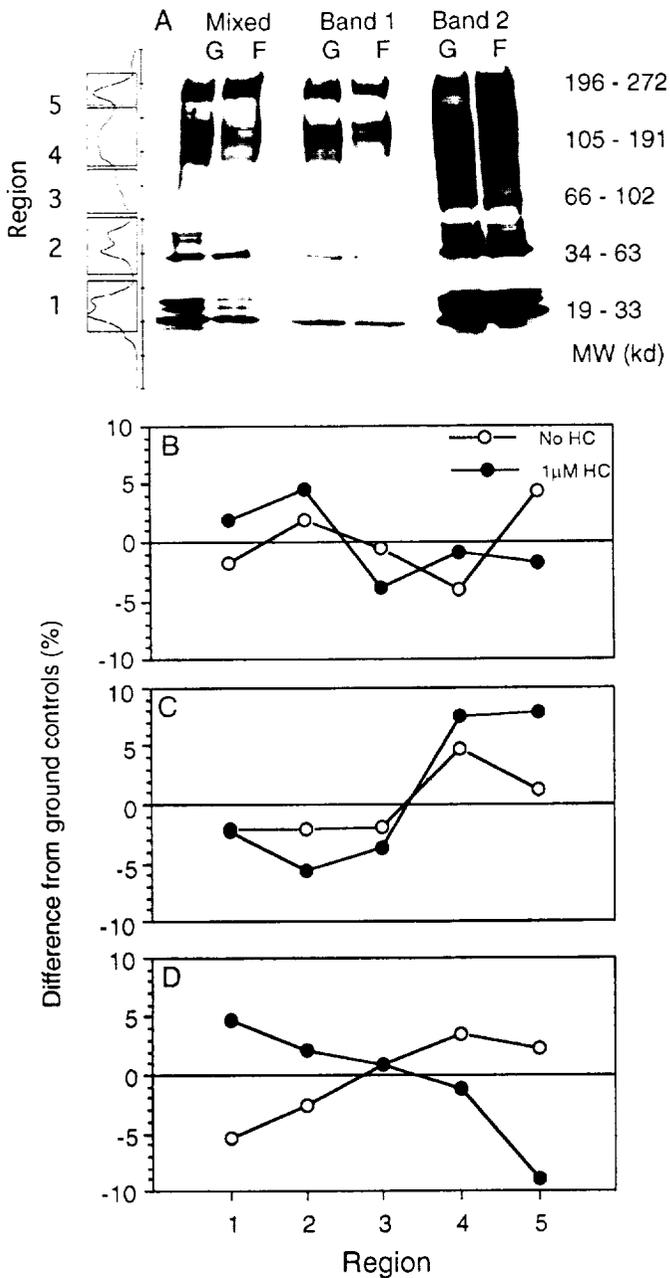


Fig. 14. A: Western blots of iGH variants extracted from cells cultured for 9 days on Earth (G) or in microgravity (F) in absence of HC. Densitometry of each lane by region (example of mixed ground sample shown) gave data for mixed (B), band 1 (C), and band 2 (D) cells.

are in combination with more dense cells; derepression would then occur when the cells were used as a subpopulation. This latter hypothesis could be tested by adding spent culture medium from one subpopulation of cells to the other.

The rationale for testing cells of two different densities is based on our early data base that shows that there are reproducible differences in the densities and activities of GH cells in the pituitaries of young adult male rats. Approximately one-half of all GH cells have densities > 1.070 g/cm³; because a majority of the other hormone-producing cell types have densities < 1.070

g/cm³, the percentage of cells in band 2 is obviously higher. Technical constraints did not permit us to further purify GH cells in band 2, but this can be accomplished to >90% by using combinations of density gradients designed to separate cells on the basis of differences in either size, density, or laser light scatter (8). Functional and biochemical differences in GH molecules released from band 2 cells in vitro include 1) greater amounts of disulfide-linked oligomeric forms (9) and 2) greater biological activity [based on the ability to promote long bone growth of the hypophysectomized rat (13, 20)]. Implantation of band 2 cells, but not band 1 cells, into the cerebral ventricles of hypophysectomized rats results in increased body weight, muscle weight, and long bone growth of the recipient. Other data also indicate that GH cells in these two density ranges respond differently to 1 μM HC (29) and 10⁻⁹ M GHRH (13).

HPLC separations of different molecular GH size classes were done to see whether our flight results could be explained at the molecular level. The data in Fig. 9 only go so far in helping to understand possible mechanism(s) at play. Several different molecular-weight variants of GH are known to be present in mammalian pituitary tissue, mammalian sera, and cell culture media (9, 23). Gel size exclusion chromatography is commonly used to display molecular heterogeneity and reports of "big-big," "big," and "normal" (monomeric, 22,000) GH abound (2). Interpretations of the physiological significance of these different high-molecular-weight forms range from the association of hormone monomers with carrier proteins to oligomerization and other posttranslational modifications. There is some agreement that high-molecular-weight iGH has less activity than does 22,000 iGH in immunoassays and selected bioassays (2); however, evidence that GH forms > 22,000 have considerable activity in the rat tibial-line bioassay is equally compelling (7). Our results suggest that the statistically significant decrease in release of iGH from mixed HC-exposed cells in microgravity (Fig. 6) can be attributed to a significant reduction in GH molecules of monomer-dimer size. The design of future spaceflight experiments with pituitary cell cultures to study the effects on monomer-dimer GH level could focus on variables of paracrine interactions between cells ± HC because these affect the GH system.

The mechanisms by which two hypothalamic peptide hormones, GHRH and somatostatin, regulate the release of GH from GH-producing cells via membrane receptors is well understood. In hypothalamic neurons of spaceflown rats, the mRNA and intracellular contents of both GHRH and somatostatin, two peptide regulators of GH cell function, are significantly suppressed (26). These same investigators have also reported that the effects were not seen in cells producing other neuroendocrine peptides, a result that implies that the entire hypothalamic-pituitary GH control axis may be particularly sensitive to microgravity. To our knowledge, this report represents the first time that the responsiveness of spaceflown pituitary cells to GHRH challenge has been studied. The results of our

RELEASED GH (No Hydrocortisone)					INTRACELLULAR GH (No Hydrocortisone)			
ASSAY	CELL	FLIGHT	POSTFLIGHT CULTURE 3 DAYS	6 DAYS	ASSAY	CELL	FLIGHT	POSTFLIGHT CULTURE 6 DAYS
EIA	Mixed				EIA	Mixed		
	Band 1					Band 1		
	Band 2					Band 2		
Bioassay	Mixed	///			Bioassay	Mixed		
	Band 1					Band 1	///	///
	Band 2					Band 2	///	///
RELEASED GH (With Hydrocortisone)					INTRACELLULAR GH (With Hydrocortisone)			
EIA	Mixed				EIA	Mixed		
	Band 1					Band 1		
	Band 2					Band 2		
Bioassay	Mixed				EIA	Mixed		
	Band 1					Band 1		
	Band 2	///	///	///		Band 2		

Fig. 15. Summary of flight effects on extracellular and intracellular GH as a function of cell sample, hormone assay, hydrocortisone, and postflight incubation. EIA, enzyme immunoassay. Responses between flight and ground are represented as not significant (open boxes), flight significantly greater than ground ($P < 0.05$; hatched boxes), and flight significantly less than ground ($P < 0.05$; solid boxes).

testing establish altered responsiveness of GH cells in all three cell samples (mixed, band 1, and band 2) to GHRH challenge after flight but shed no light on the mechanism(s) that accounts for these changes. Obviously, they could reflect changes anywhere along the signal transduction pathway. Responses of freshly dispersed GH cells to 10^{-9} M GHRH are usually robust; those seen in this study, although significant, are less so. We attribute this attenuated response to 1) the relatively long time in culture (~9 days) before testing and 2) the relatively crude culture system used. Because experimental logistics required cell processing as soon after landing as possible, the most expeditious way to conduct GHRH testing was in the spaceflown

cell-containing vials themselves. Finally, it was not possible to include GHRH in the culture medium during flight because this peptide is not stable for long periods.

The excellent morphological study by Thapar et al. (30) evaluated the rat adenohypophysis after a 7-day spaceflight by using histology, immunohistochemistry, morphometry, electron microscopy, and in situ hybridization techniques. The major effects of spaceflight were found on the morphology of adrenocorticotrophic hormone cells but not on the GH or PRL cells. Our past studies (16), as well as the present effort (Table 1), indicate that GH or PRL cell numbers are not affected by microgravity; in this respect, we are in agreement with the observations of Thapar et. al (30). However, our two studies are not in complete agreement with respect to the issue of GH or PRL "immunopositivity" after spaceflight. We find that GH-specific immunofluorescence (measured by the marker index) is two times greater in cells from spaceflown animals (16) but not in spaceflown cells themselves (Table 1). In an earlier study, which validated the flow cytometry procedures used to obtain quantitative immunohistochemical data, Hatfield and Hymer (15) pointed out that fluorescence intensity does not necessarily reflect hormone content. Indeed, Grindeland et al. (13) and Hymer et al. (16) have speculated that the unloading of spaceflight might result in conformational changes in the packaging of GH molecules that might lead to exposure of greater numbers of epitopes and therefore generate increased GH fluorescence intensity. There is a growing awareness that minor posttranslational modifications of hormone molecules immediately before exocytosis may dramatically affect hormone activity (31); such changes, as correctly pointed out by Thapar et al. (30), might very well escape detection morphologically. Why we failed to find an increased marker index in spaceflown cells (Table 1) and why Thapar et al. failed to find qualitative differences in the staining of GH cells when we had previously observed increased marker indexes of GH cells from spaceflown rats are unknown.

There are two schools of thought as to the mechanisms cells may use to detect changes in gravity levels.

Table 2. STS-46 pituitary cell culture experiment: questions and answers

Question	Answer
Does microgravity affect basal release of iGH during or after flight?	No
Does HC modify basal release of iGH during or after flight?	Usually not
Is intracellular iGH affected during or after flight?	Yes
Does HC modify that response?	Yes
Does microgravity affect basal release of bGH during or after flight?	Yes
Does HC modify basal release of bGH during or after flight?	Yes
Is intracellular bGH affected during or after flight?	Yes
Does HC modify that response?	Yes
In general, do flight-associated changes in intracellular and/or released GH (both iGH and bGH) recover after 6 days postflight?	Sometimes
Do GH cells respond to GHRH postflight?	Usually not
Does microgravity affect the amount and apparent molecular weight of extracellular and intracellular iGH; does HC affect these variables?	Sometimes
Do paracrine interactions affect GH cell function during or after flight?	Yes
Does HC modify those interactions?	Yes
Does microgravity affect biophysical changes in the GH cell?	Yes

iGH, immunoreactive GH; HC, hydrocortisone; bGH, biologically active GH; GHRH, GH-releasing hormone.

One invokes cytoplasmic organelles (e.g., cytoskeletal elements) as candidates for direct gravisensing; the other invokes physiochemical changes in the extracellular medium (e.g., decreased diffusion or microconvection) as the primary sensor (1, 22). In the latter case, a myriad of secondary intracellular changes, classified as biochemical, biophysical, or physiological in character, would ultimately explain microgravity-related changes in cell structure and function that are scattered throughout the literature. Our experiments do not define mechanisms of gravisensing by rat pituitary GH cells, but they do show that it will be possible to modify their responses to microgravity in future experiments by changing the chemistry and cellular makeup of the culture system.

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